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1. Chien, J. et al. Mol. and Cell. Endocrinology (2001) 181(1-2): 69-79
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3. Chien, J. et al. Oncogene (1999) 18(22): 3376-3382
4. Wong, E.C.C. et al. Proc. Amer. Assoc. for Cancer Res. (1997) 38: 288
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Constitutive activation of stimulatory guanine nucleotide binding protein ($G_S\alpha$QL)-mediated signaling increases invasiveness and tumorigenicity of PC-3M prostate cancer cells

J Chien[2], E Wong[3], E Nikes[1], MJ Noble[1], CG Pantazis[3] and GV Shah*[,1,2]

[1]*Department of Urologic Surgery, University of Kansas Medical Center, Kansas City, Kansas 66160, USA;* [2]*Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA;* [3]*Department of Pathology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA*

An abnormal stimulation of cAMP signaling cascade has been implicated in various human carcinomas. Since the agents activating $G_S\alpha$-mediated signaling pathways have been shown to increase *in vitro* proliferation of prostate cancer cells, present studies examined the $G_S\alpha$-mediated signaling in tumorigenicity and invasiveness of PC-3M prostate cancer cells. PC-3M cells were stably transfected with plasmids containing either wild type ($G_S\alpha$-WT) or constitutively active (*gsp* mutant of $G_S\alpha$ or $G_S\alpha$-QL) cDNAs. The stable transfectants were then tested for: (1) colony formation in soft agar; (2) cell migration and penetration of basement matrix in an *in vitro* invasion assay; and (3) the ability to form tumors and metastases in nude mice. PC-3M cells expressing $G_S\alpha$-QL protein displayed 15-fold increase in their ability to migrate and penetrate the basement membrane as compared to parental PC-3M cells or those expressing $G_S\alpha$-WT. $G_S\alpha$-QL transfectants also displayed a dramatically greater rate of growth in soft agar, and greater tumorigenicity and metastasis forming ability when orthotopically implanted in nude mice. All mice receiving PC-3M cells produced primary tumors within 5 weeks after implantation. However, the cells expressing $G_S\alpha$-QL displayed a significantly faster tumor growth as assessed by prostate weight (greater than 20-fold as compared to PC-3M cells), and produced metastases in kidneys, lymph nodes, blood vessels, bowel mesentery and intestine. Interestingly, expression of $G_S\alpha$-WT reduced the ability of PC-3M cells to form tumors in nude mice. These results suggest that persistent activation of $G_S\alpha$-mediated signaling cascade can dramatically accelerate tumorigenesis and metastasizing ability of prostate cancer cells.

Keywords: $G_S\alpha$; invasiveness; tumorigenicity; prostate cancer

Introduction

Prostate carcinoma (PC) is the leading malignancy in terms of incidence and the second leading cause of cancer deaths in men (Chen *et al.*, 1996). While a majority of PCs remain dormant for a long period of time, a significant minority of them display rapid growth and invasive characteristics (Newling, 1992, 1996). The mechanisms responsible for the latent growth of tumor in a majority of cases and for rapid progression in minority cases have not been identified. It has been suggested that tumors with greater neuroendocrine (NE) cell populations may display autonomous growth, androgen-independence and increased invasiveness (Cohen *et al.*, 1994; Huang *et al.*, 1994; Kadmon *et al.*, 1991).

Prostatic NE cells lack androgen receptors and secrete a large variety of peptides and neurotransmitters such as vasoactive intestinal peptide (VIP, bombesin, calcitonin (CT), muscarinic cholinergics, adrenergics and serotonin (Solano *et al.*, 1994, 1996; Hoosein *et al.*, 1993; Rayford *et al.*, 1997; di Sant'Agnese, 1992). Receptors for these ligands have also been localized within the prostate epithelium (Reubi, 1995; Wu *et al.*, 1996; Killam *et al.*, 1995; Gup *et al.*, 1990; Lepor and Kuhar, 1984; Abdul *et al.*, 1994; Carmena *et al.*, 1995; Aprikian *et al.*, 1996). Their mitogenic influence on the neighboring cells is documented by the findings that prostate cells in the vicinity of NE cells display increased expression of Bcl-2 and Ki-67 antigens (Segal *et al.*, 1994; van Weerden *et al.*, 1993). Since CT and VIP stimulate proliferation and/or increase invasiveness of cultured PC cells by activating stimulatory G-protein (G_S)-coupled receptors (Shah *et al.*, 1994; Solano *et al.*, 1996; Kadmon *et al.*, 1991), it is likely that $G_S\alpha$-mediated signaling may affect tumorigenicity of PC cells. Supporting this possibility are the findings that dibutyryl cAMP increases DNA synthesis, *in vitro* cell migration and invasiveness of LNCaP prostate cancer cells (Hoosein *et al.*, 1993). Since increase in local secretion of these NE ligands in aggressive PCs may cause persistent stimulation of $G_S\alpha$-coupled receptors, we examined the effects of constitutively active $G_S\alpha$ protein on invasive and tumorigenic properties of PC-3M prostate cancer cells.

Results

Characterization of $G_S\alpha$ protein expression in PC-3M cells

PC-3M cells expressing $G_S\alpha$-QL displayed greater concentrations of $G_S\alpha$-immunoreactive proteins and accumulated significantly greater levels of cAMP. The cells also displayed dramatic increase in the rate of cell

*Correspondence: GV Shah, Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, 1300 Coulter Drive, Amarillo, Texas 79106, USA
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proliferation. Although PC-3M cells expressing $G_s\alpha$-WT also displayed higher levels of $G_s\alpha$-subunit proteins (our antibody does not discriminate between mutant and wild type $G_s\alpha$ proteins) and moderately higher levels of cAMP, they did not exhibit any change in their proliferative activity. Since $G_s\alpha$-WT is identical with $G_s\alpha$-QL except for GTPase-inhibiting mutation Q227L, $G_s\alpha$-WT can serve as an appropriate vehicle control, and the changes in properties of stable $G_s\alpha$-QL transfectants can be attributed to the mutation.

Expression of $G_s\alpha$-QL increases in vitro *cell migration and invasiveness*

The results presented in Figure 1 show that $G_s\alpha$-QL transfectants exhibited a markedly greater rate of cell migration through Matrigel® than those expressing $G_s\alpha$-WT. The results were more dramatic when the conditioned medium for $G_s\alpha$-QL transfectants was used as a chemoattractant medium. Under these conditions, $G_s\alpha$-QL transfectants displayed 7–8-fold greater invasiveness than those expressing $G_s\alpha$-WT. Both cell types displayed lesser invasiveness in the complete medium, and a minimal invasion in a serum-free basal medium (containing 0.1% BSA). These results suggest that serum-derived chemoattractant(s) may be essential for invasive characteristics of PC-3M transfectants; and $G_s\alpha$-QL transfectants secrete this chemoattractant(s) in markedly greater concentrations.

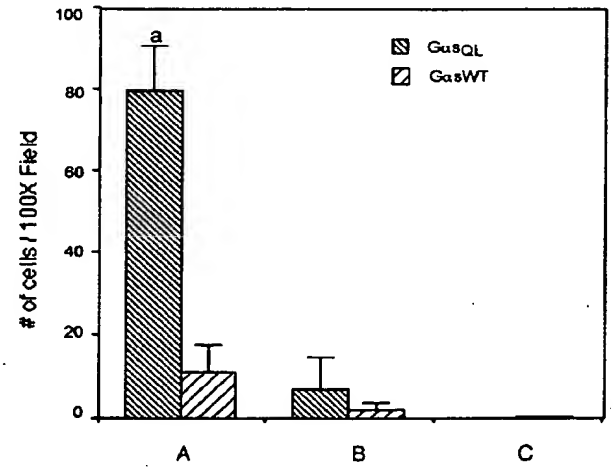


Figure 1 PC-3M cells expressing $G_s\alpha$-QL are more invasive. Invasiveness of PC-3M transfectants (expressing either $G_s\alpha$-QL or $G_s\alpha$-WT) was tested in an invasion assay described in the Materials and methods section. PC-3M transfectants could not penetrate the Matrigel when serum-free medium (as described in the Materials and methods section) was used as chemoattractant (c). Invasive ability of these cells became observable when the complete medium was used as chemoattractant (b). However, when the conditioned medium form subconfluent cultures of $G_s\alpha$-QL transfectants was added (20%) in the complete medium, the invasiveness of $G_s\alpha$-QL as well as $G_s\alpha$-WT transfectants increased by almost tenfold (a). Under all circumstances, PC-3M cells expressing $G_s\alpha$-QL displayed greater invasiveness than $G_s\alpha$-WT transfectants. These results suggest that (a) a significant phenotypical change has occurred in $G_s\alpha$-QL transfectants; (b) serum-derived factor is necessary for PC-3M cell motility; and (c) $G_s\alpha$-QL transfectants secrete this factor(s) in the conditioned medium (CM). The results are normalized data (for growth) from three independent experiments and are expressed as # of cells in 100× field (mean ± s.e.m. for $n=30$). $^*P<0.05$ (One way ANOVA and Newman-Keuls test)

$G_s\alpha$-QL increases tumorigenicity and formation of metastases

Colony formation in soft agar Normal cells must attach to a substratum to grow, whereas tumorigenic cells need not and therefore can form colonies on soft agar plates (Banerjee *et al.*, 1992). We examined whether expression of $G_s\alpha$-QL transfectants alters the ability of PC-3M cells to form colonies in soft agar. Parental PC-3M cells as well as those expressing $G_s\alpha$-WT served as concurrent controls. $G_s\alpha$-QL transfectants displayed significantly greater ability to grow in soft agar as compared to either of the two controls. The colonies formed by $G_s\alpha$-QL transfectants were observable within 4 days of inoculation, and they grew to dramatically larger size at the end of the experimental period of 16 days (Figure 2). In contrast, the colonies formed by parental PC-3M cells were not visible until after a week. These colonies were much smaller in size and fewer in number when examined at the end of a 16 day incubation. $G_s\alpha$-WT transfectants did not form visible colonies during this experimental period. Cloning efficiency of Parental PC-3M cells was $0.98\% \pm 0.034$, and the average diameter of the colonies was 10.66 ± 1.052 units ($n=69$). Expression of $G_s\alpha$-QL did not cause a significant increase in cloning efficiency of PC-3M cells ($0.74\% \pm 0.014$) ($n=57$) but caused a sevenfold increase (67.24 ± 8.855 units) in the average diameter of the colonies suggesting a dramatic increase in the ability of cells to grow in soft agar.

Tumor growth in nude mice Previous studies have shown that PC-3M cells are tumorigenic when orthotopically implanted in the prostates of athymic nude mice (Stephenson *et al.*, 1992). In consistence with these reports, all eight PC-3M-injected mice in the present study formed primary prostate tumors at the end of 5 weeks. However, only three of them formed metastases in lymph nodes during this period. No signs of metastases in other organs were observed. In all of five animals tested, $G_s\alpha$-QL transfectants formed larger primary tumors at an accelerated rate as compared to those generated by parental PC-3M cells (Figure 3). Moreover, four of these five animals displayed invasion of the tumor in blood vessels, muscles, lymphatics, bowel mesentery and kidneys. However, the tumor did not spread to the liver, lungs or brain during this period. Surprisingly, in all six animals, $G_s\alpha$-QL transfectants did not form gross tumors. The prostate weights of these animals after 5 weeks of growth were comparable with the prostates of untreated mice. However, histological examination revealed the presence of microscopic foci (Figure 4a).

Histological features in the specimens from $G_s\alpha$-QL transfectants-inoculated Nu/Nu/mice prostates are characteristic of poorly differentiated carcinoma (Figure 4b). Striking differences between parental PC-3M cells and those expressing $G_s\alpha$-QL were the rapid growth of the latter. On the other hand, $G_s\alpha$-WT transfectants displayed even slower growth than untransfected PC-3M cells (Figure 4a). After 5 weeks of growth, the PC-3M-injected control mice had only a

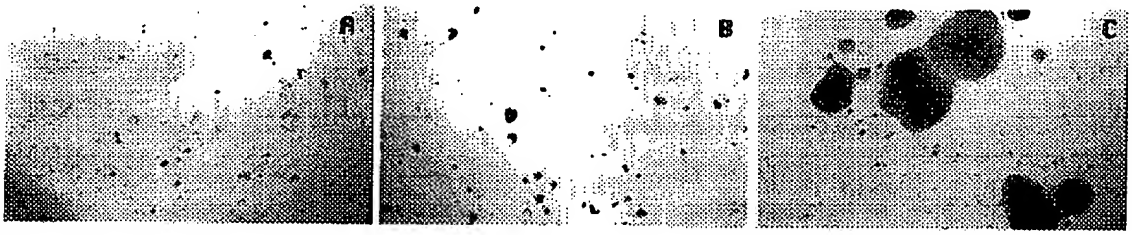


Figure 2 Expression of $G_S\alpha$-QL causes a dramatic increase in anchorage-independent growth of PC-3M cells. 5000 PC-3M cells (parental or transfected with either wild type or $G_S\alpha$-QL) were plated on soft agar as described in the Materials and methods section. The photomicrograph (100×) shows the colonies formed by the cells after 16 days. $G_S\alpha$-WT transfectants (a) did not display visible colonies. Untransfected PC-3M cells (b) displayed smaller, fewer colonies. PC-3M cells expressing $G_S\alpha$-QL formed large colonies (c) which became visible as early as within 4 days after the plating

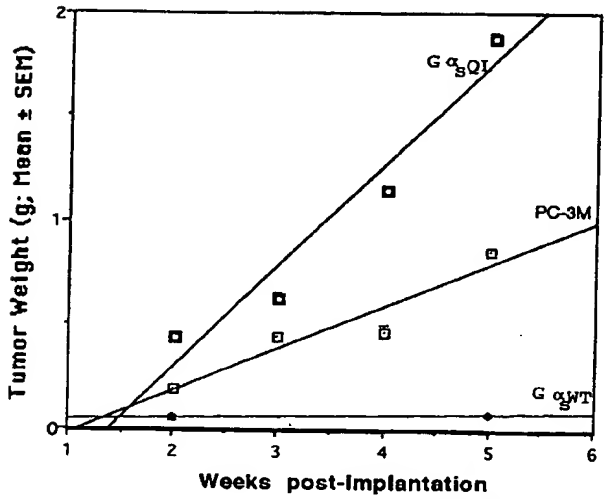


Figure 3 Expression of $G_S\alpha$-QL causes a large increase in tumorigenicity of PC-3M cells. The rate of tumor growth was fastest in mice implanted with PC-3M cells $G_S\alpha$-QL. After orthotopic implantation of PC-3M cells (as described in the Materials and methods section), the mice were sacrificed every week, their prostates were surgically removed and weighed (number of mice for each data point varied from two to five). The rate of tumor growth was fastest in the case $G_S\alpha$-QL. In contrast, the mice receiving $G_S\alpha$-WT transfectants did not display apparent increase in their prostate weights

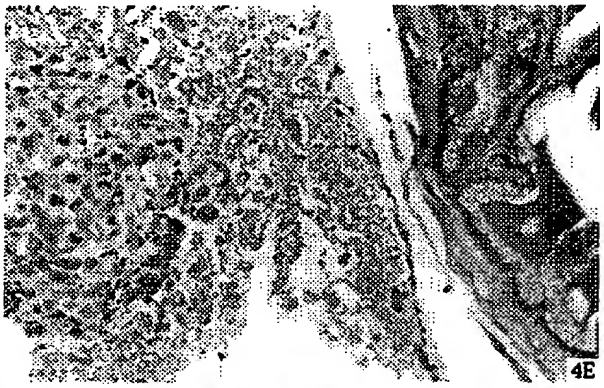
microscopic tumor of 4–5 mm in greatest dimension (Figure 4c). In contrast, mice injected with $G_S\alpha$-QL transfectants had grossly detectable tumor that has completely replaced the normal mouse prostate (Figure 4d). Other distinguishing features between the controls (either untransfected or transfected with $G_S\alpha$-WT) and $G_S\alpha$-QL transfectants-injected prostates were the cytological changes. Prostates inoculated with PC-3M and $G_S\alpha$-WT transfectants displayed large cells with abundant cytoplasm and a few small nucleoli (Figure 4e and f). Tumors formed by $G_S\alpha$-QL transfectants, on the other hand, had large cells with abundant cytoplasm and prominent macronucleoli (Figure 4g). In addition, mitoses and focal necrosis were more abundant.

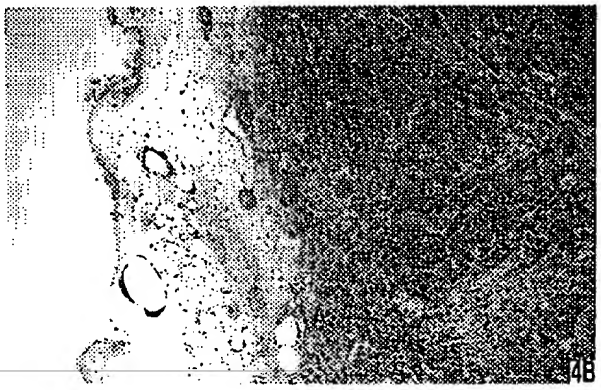
Discussion

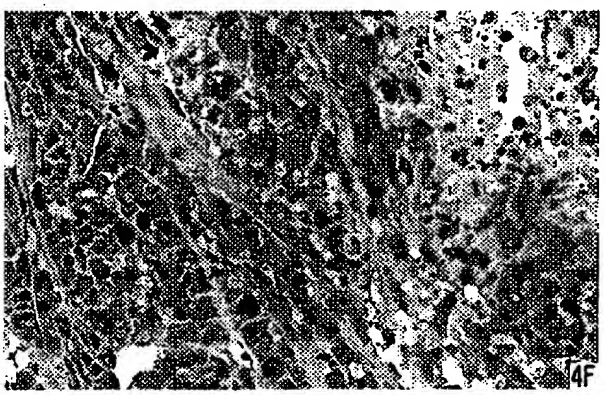
Several lines of evidence indicate that cAMP could induce a mitogenic response or increase *in vitro* invasiveness in prostate cancer LNCaP cells (Shah *et al*., 1994; Logothetis and Hoosein, 1992). There is also evidence to suggest that persistent activation of $G_S\alpha$-mediated signaling stimulate proliferation of certain cell types and can contribute to invasive tumor development in humans (Gaiddon *et al*., 1994; Muca and Vallar, 1994; Shintani *et al*., 1995; Zeiger and Norton, 1993; Drews *et al*., 1992; Reubi, 1995; Juarranz *et al*., 1994; Wu *et al*., 1996; Hoosein *et al*., 1993; Shah *et al*., 1994; Lefkowitz, 1993; Clapham, 1993). However, the role of $G_S\alpha$-mediated signal transduction in prostate tumorigenesis and tumor progression has not been investigated. The present results for the first time demonstrate that constant stimulation of the $G_S\alpha$-mediated signaling cascade could dramatically change proliferative, invasive and tumorigenic properties of prostate cancer PC-3M cells. These preliminary results also describe an animal model to study the role of signaling pathways in rapid progression of human prostate cancer.

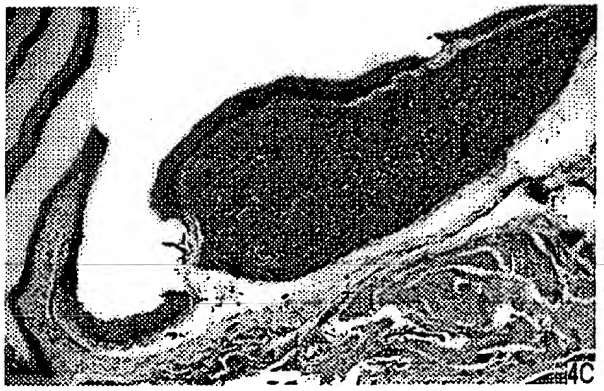
Previous studies from this laboratory have shown that PC-3M cell stably transfected with $G_S\alpha$-QL cDNA display elevated expression of $G_S\alpha$-subunit proteins; and the expressed proteins are functional as demonstrated by a large increase in cAMP accumulation. These results also suggest that an increase in cAMP accumulation alone may not be sufficient for increased cell proliferation and tumorigenicity, and may also involve nifedipine-sensitive Ca^{2+} channels.

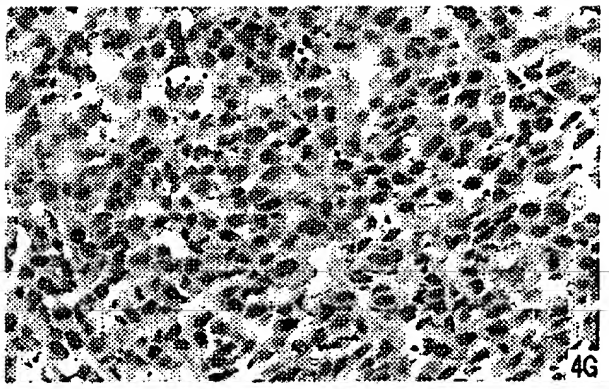
The present study extends the earlier results by presenting the evidence that expression of $G_S\alpha$-QL causes a dramatic increase in invasive and tumorigenic properties of PC-3M cells. The results from invasion assays reveal that $G_S\alpha$-QL transfectants display a large increase cell motility and invasion capacity. In contrast, those expressing $G_S\alpha$-WT do not show a similar increase. Since the invasion capacity reflects the combined effect of: (1) increased but directed proteolysis of the basement matrix; and (2) cell motility that permits migration of the cells through the degraded membrane, $G_S\alpha$-QL transfectants may have induced either or both events. Indeed, the mechanisms associated with cAMP signaling cascades have been shown to increase invasiveness of prostate and lung carcinoma cell lines (Logothetis and Hoosein, 1992; Tanaka *et al*., 1995; Young *et al*., 1995). These mechanisms have also been shown to induce expression of the urokinase type plasmalogen activator gene as well as its receptor gene, a major component of invasion (Langer *et al*., 1993; Miray-Lopez *et al*., 1992). Additional studies are underway to test whether











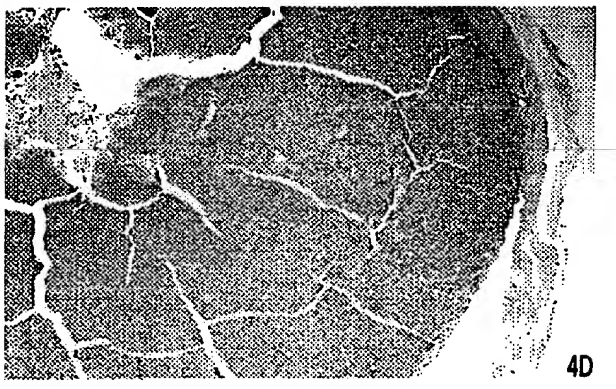


Figure 4 Histological sections of prostate tumors formed by parental and transfected PC-3M cells. (a) 40 × H&E section of prostate transplanted with PC-3M cells expressing $G_{s}\alpha$-WT in a Nu/Nu mouse (5 weeks of growth). (b) 40 × H&E section of a tumor formed by $G_{s}\alpha$-QL transfectants when transplanted in a Nu/Nu mouse prostate (5 weeks of growth). (c) 40 × H&E section of tumor formed by parental PC-3M cells in a Nu/Nu mouse prostate (5 weeks of growth). (d) 4 × section of tumor formed by $G_{s}\alpha$-QL transfectants when implanted in a Nu/Nu mouse prostate (5 weeks of growth). Note that the tumor has completely replaced mouse prostate. (e) Same section as (a) but at 400 ×, note the large cells with little acinar differentiation. (f) Same section as (c) but at 400 ×, note the large cells with little acinar differentiation, prominent macronucleoli and necrosis. (g) Same section as (b) but at 400 ×, note the large cells with prominent macro nucleoli and mitoses

8-bromo cAMP induces a similar increase in these paradigms and that the dominant negative mutant of $G_s\alpha$ reverses these changes.

$G_s\alpha$-QL transfectants also displayed a dramatic increase in their ability to grow in soft agar. The colonies formed by these cells were several-fold larger in diameter than those formed by parental PC-3M cells. Interestingly, $G_s\alpha$-WT transfectants did not form any visible colonies during the experimental period. Another major difference between $G_s\alpha$-QL transfectants and parental cells was the rapidity of colony formation. While colonies formed by the cells expressing $G_s\alpha$-QL transfectants were visible within 4 days of inoculation, those formed by parental cells could be seen only after a week. Since anchorage-independent growth and ability to invade and migrate through basement membrane are essential components of the metastatic process, we also tested whether $G_s\alpha$-induced increase in anchorage-independent growth and cell motility would translate into faster tumor growth and formation of distant metastases in athymic nude mice. The results from nude mice experiments reveal that expression of constitutively active $G_s\alpha$ not only caused a marked increase in the ability of PC-3M cells to form tumors, but also accelerated their growth rate and metastasizing capacity. $G_s\alpha$-QL transfectants replaced the whole mouse prostate within 5 weeks, invaded locally in blood vessels and muscle and also formed distant metastases in lymph nodes and several visceral organs. In contrast, parental PC-3M cells formed smaller tumors that were mostly confined within the prostate. A few of them formed metastases in lymph nodes but there was no incidence of metastases in any other organs. Our results from parental PC-3M cells are in complete agreement with the previous large study (Stephenson *et al.*, 1992), where orthotopic implantation of PC-3M cells was shown to produce primary tumors in athymic mice, some incidence of metastases in lymphatics, but no evidence of metastases in other organs. A selective ability of $G_s\alpha$-QL transfectants to rapidly invade mouse prostates and other organs may have been the result of $G_s\alpha$-induced changes in secretory and invasive characteristics of PC-3M cell. However, it is also possible that these changes invasiveness are secondary to $G_s\alpha$-QL-induced changes in PC-3M cell phenotype.

The formation of metastases in a host organ may depend on: (1) the ability of tumor cells to escape the primary site and migrate to a new organ(s); and (2) the ability of the tumor cells to implant, survive and grow in a new environment. While invasive characteristics of a cell may depend on its intrinsic machinery, the later characteristics may involve host-tumor interactions. It is conceivable that GTPase-deficient $G_s\alpha$ ($G_s\alpha$-QL) may promote metastases by increasing invasiveness of the tumor cells as well as by stimulating autocrine/paracrine factors necessary to facilitate their implantation and sustain their growth in a host organ. Present results for the first time demonstrate a role for GTPase-deficient $G_s\alpha$ in tumorigenicity and in the metastasis-forming ability of PC-3M prostate cancer cells.

In contrast to tumor promoting actions of GTPase-deficient $G_s\alpha$, $G_s\alpha$-WT caused a reduction in tumorigenicity of PC-3M cells. PC-3M cells expressing $G_s\alpha$-WT did not form any macroscopic tumors. Only microscopic foci in apparently normal mouse prostates were visible. It is conceivable that wild type $G_s\alpha$ may selectively activate adenylyl cyclase and other mechanism(s). but not mitogenic signaling; and increased cAMP may then attenuate mitogenic signaling by suppressing the raf-1-MAP kinase pathway (Faure and Bourne, 1995). Alternatively, increased concentrations of wild type $G_s\alpha$ may sequester other signaling components such as GDP/GTP exchange proteins, and their depletion could lead to attenuation of mitogenic signaling events and reduced tumor growth.

The presence of the *gsp* Oncogene, that express GTPase-deficient form of $G_s\alpha$ protein in human diseases, has not been reported in PC (Gaiddon *et al.*, 1994). However, a relatively greater population of NE cells in malignant prostates could also provide an abundant supply of ligands activating $G_s\alpha$-coupled receptors (Cohen *et al.*, 1994; Huang *et al.*, 1994; Aprikian *et al.*, 1994). The present results suggest that persistent stimulation of $G_s\alpha$-mediated signaling could dramatically accelerate prostate tumor growth and formation of metastases. The mechanisms by which GTPase-deficient $G_s\alpha$ accelerates prostate tumor growth and progression remain to be investigated. Activated $G_s\alpha$ has been shown to stimulate adenylyl cyclase activity, activate voltage-gated calcium channels and the MAP kinase signaling cascade and may stimulate PI-3 kinase activity in certain cell types (Lopez-Ilasaca *et al.*, 1997; Murga *et al.*, 1998). Since tumor progression involves multiple events such as cell proliferation, directed protease activation, cell migration, interaction with extracellular matrix and other related events, it is conceivable that constitutive activation of $G_s\alpha$ may induce several of these events by activating multiple signaling cascades. Recent results from this laboratory that $G_s\alpha$-induced DNA synthesis in PC-3M cells could be attenuated and abolished by nifedipine implicates the role of dihydropyridine-sensitive calcium channels in PC-3M cell proliferation (companion MS1). Similarly, a role for cAMP-dependent protein kinase A in cell proliferation and invasive capacity of prostate cancer LNCaP cells as well as thyroid, pituitary and lung carcinoma cells has also been reported (Hoosein *et al.*, 1993; Shah *et al.*, 1994; Young *et al.*, 1995; Logothetis *et al.*, 1994). However, the specific events associated with $G_s\alpha$-mediated tumor progression remain to be elucidated.

In summary, we presented a human prostate cancer metastatic model using genetically altered PC-3M cells. By activating the $G_s\alpha$-mediated signaling cascade, we have shown a dramatic increase in tumorigenic properties of PC-3M cells and formation of metastases in lymph nodes and various soft tissues within a period of 5 weeks. These preliminary results demonstrate the importance of $G_s\alpha$-mediated signaling pathways in tumor progression of androgen-independent PC.

Materials and methods

Cell culture

Dr Isiah Fidler (Anderson Cancer Center, Houston, TX, USA) kindly provided PC-3M cell line, a variant of PC-3 cells. The cells were maintained in the complete medium (RPMI 1640, 15% horse serum, and 5% fetal calf serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin). Stable

transfectants were maintained in the complete medium supplemented with 400 μg G418/ml.

DNA constructs

Complementary DNA for wild type ($G_s\alpha$-WT) and constitutively active ($G_s\alpha$-QL) $G_s\alpha$ subunits were provided by Dr R Iyengar (Department of Pharmacology, Mount Sinai Medical Center, NY, USA). Additional details regarding the construct have been described (see the companion MS1).

Transfection, generation of stable transfectants and their characterization

Plasmid DNA transfection of PC-3M cells was performed as described previously. The stable transfectant cell lines were obtained by neomycin selection of 3–4 weeks (complete medium containing 400 μg/ml of G418^{res}) and examined for $G_s\alpha$ expression by (i) immunodetection of $G_s\alpha$ subunit using Western analysis; and (ii) by the measurement of cAMP accumulation. The procedures are described in detail in the companion MS1.

Soft agar assay

Complete medium containing 1% low-melting point temperature agarose was poured into six well plates (2 ml per well) and allowed to solidify at 4°C to form a bottom layer. PC-3M cells (5×10^3 per well; parental as well as those expressing either $G_s\alpha$-WT or $G_s\alpha$-QL) were mixed in complete medium with 0.5% agarose and seeded as a top layer. The agarose was solidified at 4°C and then incubated at 37°C. On day 16, the colonies were stained with 1 ml of PBS containing 0.5 mg per ml piodonitrotetrazolium violet, which is converted into colored product by live cells only. The colonies with greater than 50 cells were counted.

Invasion assay

The experiment used six well Matrigel two-tier invasion chambers (Collaborative Biomedical Products, Bedford, MA, USA). PC-3M cells (2.5×10^5 cells per well; expressing either $G_s\alpha$-QL or $G_s\alpha$-WT) were seeded in the upper insert in a serum-free basal medium (RPMI 1640 medium containing 0.1% BSA, 150 mg/ml of G418, 4 mM L-glutamine, 100 μg/ml penicillin G and 100 μg/ml streptomycin). The lower chamber contained chemoattractant medium consisting of 80% complete medium and 20% conditioned medium obtained from subconfluent cultures of $G_s\alpha$-QL transfectants. The incubations were carried out for 48 h. At the end of this period, upper inserts were removed, and the layer of Matrigel (inside bottom of the upper insert) was scraped off using cotton swabs, fixed and stained using Diff Quick staining (Dade Diagnostics, Aguar, PR). The invasive cells would penetrate through the Matrigel layer and would be on the outside bottom of the upper insert. The numbers of cells on the outside bottom of the upper insert were counted. At least six 100× fields per inserted were examined. The data from invasion assay was corrected for cell growth during experimental period as follows: the experimental cells were plated at a density of 10^5 cells per well in six well dishes in chemoattractant medium and increase in cell number was determined after 48 h. The invasive potential was normalized relative to the cell line/chemoattractant medium combination that initially resulted in the lowest average number of cells per 100× field. All experiments were done in triplicate and the results are expressed as mean ± s.e.m.

In addition to comparing the invasive potential of the two cell types (PC-3M cells expressing either $G_s\alpha$-QL or $G_s\alpha$-WT), chemoattractant properties of serum-free basal medium (containing 150 mg/ml of G418 sulfate) and conditioned medium obtained from PC-3M cells expressing $G_s\alpha$-WT were also tested in the invasion assay.

Tumor growth in Balb/c Nu/Nu mice

Athymic Balb/c nude (Nu/Nu) mice (6–8-weeks-old) were obtained from Harlan (Milwaukee, WI, USA) and were housed two to a cage in a pathogen-free facility on a 12/12 h light/dark cycle with free access to autoclaved food and water. Sterile gowns, gloves and masks were used for handling the animals. Mice were sacrificed by decapitation under ketamine anesthesia (KU animal protocol 91-06-07-00 for surgery and euthanasia).

PC-3M cells (parental cells, or those expressing either wild type of $G_s\alpha$-QL; 1×10^6 cells in 20 μl Hank's Balanced Salt Solution) were orthotopically injected into dorsal-lateral lobes of prostates as described previously (Stephenson *et al*., 1992). Five to eight mice were included in each treatment group. The mice were then monitored for tumor growth and distant metastases in lymph nodes by palpitation. The necropsy was performed 5 weeks after the implantation, and their prostate glands as well as other organs were examined thoroughly for primary tumors and metastases. The prostate glands were dissected out and weighed, and metastases were confirmed by histology.

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